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The long-term effects of ovariectomy on bone metabolism in sheep

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Ilonka Sigrist

Tierärztin

von Sarnen OW

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PD Dr.med.vet. Brigitte von Rechenberg, Referentin
Prof. Dr. sc. techn. Erich Schneider, Korreferent

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Ilonka M Sigrist [1], Christian Gerhardt [1], Mauro Alini [1], Erich Schneider [1] and Marcus Egermann [1, 2]

(1) AO Research Institute, Davos, Switzerland
(2) Department of Orthopaedic Surgery, University of Heidelberg, Germany

Correspondence:
Marcus Egermann
AO Research Institute
Clavadelerstrasse
CH-7270 Davos, Switzerland
Phone: ++41-81-414 24 45
Fax: ++41-81-414 22 88
marcus.egermann@aofoundation.org

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Abstract

Osteoporosis and associated fractures are major public health concerns, and as such, require appropriate large animal models in order to further our understanding of this disease. Although sheep appear to be an ideal model with which to study bone loss due to estrogen depletion, limited data is available concerning the long-term effect of ovariectomy on bone in sheep. The goal of the present study was to observe the ovariectomy induced changes in bone mass, structure and metabolism in sheep over a period of 18 months. Six ewes were ovariectomized (ovx) and compared to an age-matched control group by analyzing bone mineral density, trabecular structure, biochemical markers of bone formation and resorption, and plasma estrogen levels. Bone loss (13%, $p<0.01$) occurred during the first 4 months after surgery, then stabilized and returned back to pre-ovx levels for the remainder of the study. Trabecular architecture was also altered and tended towards osteopenia with recovery to baseline values. Marker of bone formation and resorption were elevated up to 6 months post ovariectomy after which time levels returned to baseline values. Although estradiol-measurements demonstrated a clear decline following surgical ovariectomy, levels returned to normal after 6 months. Therefore, the detrimental effect of ovariectomy on sheep bone metabolism seems to be reversible with normal bone parameters being re-established within 6 months after surgery. These data seems to indicate that the sheep is an inappropriate model for human post-menopausal osteoporosis.

Introduction

Due to the dramatic increase in the elderly population and the rise in the incidence of fractures, osteoporosis has become a major public health concern [1-3]. Since new treatment strategies and better fixation methods in such fragile bones can not be developed *in vitro*, there is a great need for suitable animal models which closely simulate osteoporosis in humans [4].

Various experimental models of human osteoporosis have been described in several different species [4]. The ovariectomized rat is probably the most widely studied rodent osteoporotic model. This model mimics postmenopausal cancellous bone loss over a relatively short time period [5;6]. However, extended studies over 12 months have demonstrated increased bone mineral content, bone area and body weight, which is not comparable to the human situation [7;8]. Furthermore, the lack of the Haversian system in cortical bone, the absence of impaired osteoblast function during late stages of estrogen deficiency and the absence of multicellular unit-based remodelling in young rats also limits this model [9]. An additional requirement of experimental osteoporosis models is the ability to assess the performance of surgical implants. Such procedures are clearly impractical in the experimental rat model due to limitations in size. The “FDA Guidelines for Preclinical and Clinical Evaluation of Agents Used in the Treatment of Prevention of Postmenopausal Osteoporosis” recommend agent evaluation in two different species including ovariectomised rats and one non-rodent large animal model [10]. Although these guidelines primarily pertain to pharmaceutical drugs, pre-clinical evaluation is also required for new surgical treatment options.

Therefore, there is an obvious need for large as well as small animal models of osteoporosis.

Large animal osteoporosis models include non-human primates, dogs and sheep. Monkeys such as baboons and macaques have an advantage over other osteoporosis models due to their close physiological resemblance to humans, including gastrointestinal tract, endocrine system and bone metabolism [11-13]. Female monkeys have hormonal patterns and a monthly cycle very similar to those of humans. Aged macaques and baboons experience a similar decline in ovarian function and irregular menstrual cycles as in peri- and postmenopausal women. However, due to ethical reasons and handling costs, the experimental use of these species is rather limited.

Dogs possess cortical bone with a Haversian system and manifest internal cortical and cancellous bone remodelling processes similar to those in humans. Studies have evaluated the effects of ovariectomy on histomorphometric variables in cancellous and cortical bone in several breeds of dog [14-19]. Although significant changes in cancellous bone volume were observed in these studies, the data is conflicting and suggests that canine experimental osteoporosis may not be the most suitable animal model for studying bone loss following ovariectomy. More importantly, fracture rates in aged, sedentary dogs without ovaries and uteri are not comparable to those of postmenopausal women [20]. Finally, the emotional attachment to dogs, along with the stringent legal housing requirements, contributes to the difficulties in using this experimental model of osteoporosis.

Sheep have been extensively studied as an experimental model of bone disease [4]. Bone loss in sheep is associated with estrogen deficiency and their hormone profiles are similar to those of women [21-24]. Their size permits the implantation

of prosthetics, isolation of large quantities of fluid samples, as well as iliac crest biopsies [25]. Although reports have not described consistent decreases in bone mineral density or bone mass at different anatomical locations, significant changes in these parameters have been demonstrated in distal radius and spine [26]. Biochemical markers of bone formation such as bone-specific alkaline phosphatase were reported to increase in sheep after ovariectomy [24], indicating an increase in bone turnover similar to the human post-menopausal situation. However, only a 10% reduction in sheep bone mass has been attained through old age or estradiol privation following ovariectomy, while in osteoporotic humans, this value is at least four fold higher [27]. Reports describing the long-term effects of ovariectomy in sheep are limited and studies have predominantly focused on animals post mortem or in large time intervals [22]. Furthermore, the reported effects of ovariectomy on estrogen levels in ewes are highly controversial [28;29].

In the present study, we examine the long-term effects of ovariectomy on bone density, structure and metabolism in sheep. Multiple parameters of bone turnover including mineral density (pQCT), 3-D trabecular architecture and biochemical markers are investigated and their association with estradiol hormone levels determined.

Materials and methods

Study design

The European and Swiss laws on animal experimentation were strictly observed during the entire study and the animal research protocol was approved by the competent ethical committee. Fourteen female white alpine sheep with mean age of 3.1 ± 0.7 years at the beginning of the study were included in this study. They were divided into 2 groups based on BMD and body weight, so that there would be no statistical differences between the means. Six sheep were ovariectomized (ovx), while the others (n=8) were used as a control group (control).

The sheep were hold in group boxes under standardized conditions and natural light, and got their body weight measured weekly. During the entire observation period the animals were fed twice daily with silage (1.5 kg/animal*day)(10.3g/kg Calcium, 4.1 g/kg phosphorus), hay (0.25 kg/animal*day)(6.3 g/kg Calcium, 2.8 g/kg phosphorus) and straw as well as water ad libidum. Urine and venous blood samples were collected on day 28, 14 and 1 before surgery, and every fourth week after surgery for quantification of bone markers and hormones. The BMD was measured at distal radius by pQCT 2 weeks before surgery and at 3 months intervals after surgery. For micro-CT-measurements, bicortical iliac crest biopsies have been taken at the time of the ovariectomy and at 3, 6 and 9 months after surgery. The study ended 18 months after surgery.

Surgical procedures

In April 2003, 6 ewes (treatment group) were subjected in supine position to midventral laparatomy under general anaesthesia with Isofluran (Halocarbon Laboratories, River Edge, NJ) and intravenous Temgesic (Buprenorphine HCl 0.3

mg/mL, Reckitt& Colman Pharmaceutics, Hull, UK). Bilateral ovariectomy was performed, with care being taken to ligate doubly the ovarian ligament, artery and vein. Post-operative pain medication was given as necessary.

Bicortical transiliacal biopsies were taken under general anaesthesia in all 14 animals bedded in prone position. To cut them out, a trephine drill (TL 05 trephine 2.0, external diameter 7.3 mm, internal diameter 6.4 mm) under constant irrigation has been used. The length of the biopsies was given by anatomical conditions, as the transfixion was made 1 cm below the iliac crest, alternating medial and lateral from the spina iliaca, either on the right or on the left pelvic bone.

BMD-measurement

The bone mineral density (BMD in grams per cubic centimetre) was measured by pQCT using a Densiscan 1000 (SCANCO Medical AG, Bassersdorf, Switzerland). The distal radius of both sides was examined. To avoid artefacts caused by movement, the measurements were performed under general anaesthesia. After fixation of the limb in the CT scanner, a projectional scout view was accomplished to determine the axial position. The joint surface was defined as starting point using a reference line. A highly filtered low-energy X-ray source was used to enhance the bone contrast. The effective energy of the X-ray beam was always set to forty kilo-electron volts and 0.5 mill modus. The slice thickness was 1 mm and a matrix of 512x512 pixels was used resulting in a spatial resolution of 290x290µm. Ten consecutive slices were obtained, and a contour surrounding the radius on each cross section was detected. The µ50-CT value was calculated using the interior 50% of a section to ensure that only cancellous bone was measured.

Structural parameters in micro-CT

After harvesting transiliacal biopsies and immediate fixation in 70% Ethanol, micro-CT scans using MicroCT 40 (SCANCO Medical AG, Bassersdorf, Switzerland) were performed. This system is working with a fan-beam in a multislice mode. An X-ray tube with microfocus is used as source, and a CCD-array as detector. The spatial resolution was 22 µm in all directions. The volume of interest included a cube with a side length of 4 mm and was displayed in 14x14x14 cubic micron voxels. Bone volume/ total volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp) and the degree of anisotropy (DA) were measured.

Bone markers

Venous blood samples were allowed to clot for 30 minutes at room temperature. The serum then was collected by centrifugation (30 minutes at 4000 rpm, room temperature). Serum samples were stored at -20°C until assay.

Bone specific alkaline phosphatase (bALP)

Serum bALP levels were determined by ELISA, using the METRA BAP EIA kit (TECOmedical AG,5034 Suhr CH). Principle of the procedure: Metra BAP is an immunoassay in a microtiter strip format utilizing a monoclonal anti-BAP antibody coated on the strip to capture BAP in the sample. The enzyme activity of the captured BAP is detected with a pNPP substrate. Control samples were within the given range (14.9 and 15.4 U/L for low control (range 9.8 – 21.1 U/L); 64.3 and 86.6 U/L for high control (range 62.0 – 97.2U/L).

Deoxypyridinoline (DPD) crosslinks

As no reliable method to measure DPD in sheep urine was found except for the HPLC method, which is very time-consuming and laborious for such a big amount

of samples, the METRA DPD ELISA has been used. Finally HPLC and DPD ELISA have been compared in a parallel measurement with a few samples showing significant relationship. The METRA DPD ELISA is a competitive enzyme immunoassay (TECOmedical AG, Suhr CH). DPD in the sample competes with conjugated DPD-alkaline phosphatase for the antibody and the reaction is detected with a pNPP substrate. Control samples were measured as 16.8 and 15.1 nmol/L for low control (range 13.1 – 21.4 nmol/L) and 86.2 and 92.2 nmol/L for high control (range: 71.0 -115.9 nmol/L). The analysis using the ELISA-method was verified by high pressure liquid chromatographie. The DPD results were corrected for urinary concentration by creatinine, which has been quantified by the METRA Creatinine assay from TECOmedical AG (Suhr CH) basing on a quantitative, colorimetric assay (modified Jaffé method).

Pyridinoline (PYD)

For a quantitative measure of the excretion of pyridinoline crosslinks in serum, the METRA Serum PYD EIA kit (TECOmedical AG, Suhr CH), a competitive enzyme immunoassay in a microtiter plate, was used. PYD in the samples or standards competes with PYD immobilized on the plate for polyclonal rabbit anti-PYD antibody. Bound antibody is detected by goat anti-rabbit antibody conjugated to alkaline phosphatase and the reaction is detected with pNPP substrate. Control samples were measured as 2.0 and 2.3 nmol/L for low control (range 1.6. – 2.6 nmol/L) and 6.5 and 8.1 nmol/L for high control (range: 6.3 - 10.4)nmol/L).

Hormone Estradiol (E2)

Serum estradiol levels were determined with the 17β-Estradiol ELISA from IBL Hamburg (Germany). Principle of the procedure: an unknown amount of estradiol present in the sample and a fixed amount of estradiol conjugated with horse-

radish peroxidase compete for the binding sites of a polyclonal estradiol antiserum coated onto the wells. Manufacturer´s protocol was followed as described and samples were assayed in duplicates. The intra- and inter-assay coefficients of variation and the limits of detection were 4.9-5.9%, 6.2-10.1% and 3 pg/ml respectively. All values below the detectable limit were recorded as 0 pg/ml. The controls were within the given ranges (low control: 115 and 101 pg/ml vs. 50 – 130 pg/ml, high control: 384 and 218 pg/m vs. 168 - 432 pg/ml).

In addition to the method mentioned above, the serum estradiol-levels were determined at an external, independent laboratory (Diavet, Bäch CH) with extensive experiences in biochemical veterinary diagnostics. 17β-Estradiol was measured using an electrochemi-luminescent-sandwich-ELISA.

Statistical analysis

Statistical analysis was performed using SPSS software package (SPSS Inc., Chicago, USA). Results are presented in mean ± standard deviation. For comparison between the groups analysis of variance (ANOVA) was used for bone markers and non-parametric testing (Mann-Whitney test) was used for others. The level of significance was set at 0.05.

Results

All animals tolerated the surgical procedures well without any side effects. One ovariectomized sheep died at 16 months of observation due to oesophageal infection which was not related to the treatment methods of this study. During the entire observation period, the mean body weight was 67.6 ± 4.5 kg for control and 67.5 ± 5.3 kg for ovx. The body weight fluctuated over time but the individual changes were below 10% in all sheep. No differences at any time point were found between the groups.

Bone Mineral Density

The cancellous bone mineral density at distal radius in control sheep remained constant with time at ~ 0.71 g/cm3 out to 17 months. The course of cancellous bone mineral density in the control group showed small changes with a slight decrease at the beginning of the study followed by minimal increase at 15 months. All changes were not significant compared to baseline. Ovx sheep exhibited an rapid, initial phase of cancellous bone loss of -12.7% (p=0.008) between 0 and 4 months post ovariectomy. Then, a period of relative stabilisation of cancellous bone volume at this osteopenic level occurred between 4 to 12 months, followed by a phase of minimal increases in bone mass until 17 months post surgery (Figure 1).

Static bone morphometry and trabecular architecture by micro-CT

The changes of the structural parameter in cancellous bone of the iliac crest are shown in Figure 2. Following ovariectomy, the parameters bone volume to total volume (BV/TV) (3 months: p=0.007, 6 months p=0.010) and trabecular thickness (3 months: p=0.010) are decreasing up to 6 months with a subsequent increase to

baseline values. Trabecular number and separation did not change during the observation period.

Bone markers

Figure 3 depicts the serum bALP patterns over the first phase (6 months) of observation. The control group shows seasonal variations which have to be considered, while in the ovx group, there is a 50% elevation of bALP at 2 months after ovariectomy ($p=0.015$). In this group bALP decreases until fall towards baseline values, but still elevated at 6 months compared to control group ($p=0.006$). No differences were found at any later time point. Increased levels of serum pyridinoline were found at 2 months after ovariectomy only ($p=0.005$) and no differences between the groups were determined at any later time point until 18 months. The urine deoxypyridinoline values did not show significant differences between the controls and the ovariectomised sheep.

Estradiol

17ß-Estradiol decreased 3 months after ovariectomy ($p=0.021$) in the treated group to values below the detection limit (3pg/mL) and then increased massively in both groups. The developing of the control values showed a period of anoestrus in May and June, followed by the breeding season in fall and winter with higher serum estradiol levels (mean 20.8 pg/mL) (Figure 4A). With the exception of significant decrease of estrogen levels following ovariectomy no differences were found between the two groups (Figure 4B). The data were validated using a different method at an independent laboratory.

Discussion

This study investigated the long term effect of ovariectomy on ewe, demonstrating an early bone loss (-13%) followed by rebound effect in the later phase of the observation period.

To investigate postmenopausal bone loss and its consequences, there is a great need to develop an osteoporotic animal model for the testing of orthopedic implants as well as for pharmacological research on prevention and treatment. Sheep show several advantages as a model for osteoporosis: easy handling and keeping, availability in large numbers so that large-scale studies are possible, implant dimensions comparable to human implants, similar metabolic rate and bone remodelling process as humans [4;25], and osteopenic response to menopause simulating manipulations [24].

The present study evaluated the effect of ovariectomy on bone metabolism in sheep by determining bone mass, bone micro architecture, biochemical markers of bone formation and resorption as well as estradiol. Dietary effects were excluded by standardized feeding and a bias due to physical activity was constrained by single group husbandry. The surgery was performed in spring when lowest bone mass is usually observed during the season to minimize seasonal influences on the bone loss following ovariectomy. As ovariectomy is a standard procedure in our institution [30], the authors omitted a sham group to avoid the use of more animals in the study as requested by the ethical committee, although all sheep had bilateral transiliacal biopsies. Therefore, a limitation of this study is the lack of a true sham operated control group since the effect of ovariectomy can not be distinguished from any influences of wound healing. Prior

studies have demonstrated an increased bone turnover following ovariectomy in sheep leading to a loss of bone mass of approximately 10%. Geusens et al. observed a non significant bone loss of -3 to -9 % at the femur [31], Lill et al. measured a decrease of 5.5% of BMD at distal radius in ovariectomized sheep with a calcium-wasting diet [30], while several publications from Turner et al. report about a decline of BMD from 3 to 8 % [20;24;25]. However, it has not been determined whether these small ruminants experience a short-term effect of ovariectomy, or if they develop osteoporosis over a longer period of time as it is the case in humans. In the present study, the great majority of bone loss (-13%) occurred during the first 4 months post ovariectomy. This initial, rapid phase was followed by a period between 7 to 9 months during which cancellous bone mass appeared to stabilize at the mentioned osteopenic level. Afterwards the bone mass seemed to increase towards baseline values. The changes of bone mass in control group are due to seasonal changes [20]. During the period between 7 and 14 months, both bone loss and bone turnover subsided. The rapid, initial phase of bone loss is coincident with the maximal increase in bone turnover, which confirms previously reported studies [32]. These data clearly suggest short term effects on bone mass in ovariectomized ewes.

Several other studies have been determining BMD using DXA, either on spine, femur, calcaneus or distal radius, with different results [23;24]. BMD measurements by DXA (a two dimensional technique) are affected by skeletal size, and changes in density may be obscured by the mass of wool, soiling of the skin and subcutaneous fat layer [33]. Measurement of volumetric BMD by pQCT though is advantageous as it is independent of skeletal size; it allows for selective measurement of cortical and trabecular density and bone area [34].

As many recent studies have shown that BMD alone is not sufficient to determine the strength of cancellous bone, we also looked at static bone morphometry and trabecular architecture using microcomputed tomography [35;36]. Those parameters are important factors in determining bone strength and the risk of fractures. Micro computed tomography (micro-CT) enables the complete three-dimensional digitisation of small specimens at resolutions of between 10 and 75 microns. It has the ability to provide quantifiable measures of bone architecture and morphology in three dimensions, giving the exact structure of a bone biopsie. The decrease in BV/TV goes along with a decrease of trabecular thickness indicating. Although the loss of bone mass in post-menopausal women is driven by decrease of trabecular number [37], the results are similar to previous reports [26] and indicate the increased bone resorption and thinning of the trabecular structures.

Bone-specific alkaline phosphatase (bALP) has been analysed to measure bone formation. While the control group shows seasonal fluctuations, an elevation of serum bALP-levels until 6 months post ovariectomy can be observed indicating an increased bone turnover. No differences between the groups were observed at any time point later than six months. Turner et al. [24] as well as Chavassieux et al. [21] also describe a high bone turnover after ovariectomy. Similar to our results a trend towards decreasing levels until the end of the study (6 months) can be found. However in both publications, the seasonal timepoint of the ovariectomy is unknown.

To show bone resorption, the pyridinoline crosslinks deoxypyridinoline (DPD) and pyridinoline (PYD) have been quantified. Those crosslinks are formed during the process of collagen maturation between the chains, and as bone collagen

undergoes a higher turnover rate than other tissue sources of collagen, they are specific markers for bone resorption. We found increased levels of bone resorption marker at 2 months after ovariectomy only. Chavassieux et al. [21] observed the highest increase of type 1 collagen C-telopeptide at 3 months after ovariectomy.

By removing the ovaries, the major source of estrogens is eliminated to simulate as much as possible the situation after menopause in women. Very few authors of studies about ovariectomy have been testing the estradiol levels of the animals after operation. Johnson et al. writes that ovariectomy does not totally eliminate 17-β-estradiol synthesis in sheep – values between 4 and 6 pg/mL- [28], while Karsch et al. postulates that serum estradiol concentrations in intact anestrous ewes averages 1 pg/mL, and that estradiol levels in ovariectomized animals are well below the limit of detection of any biochemical assay [29]. As the opinions over this topic are divergent and not much literature can be found, estradiol has been quantified in all animals of this study. The results show a significant drop in systemic estrogen levels due to ovariectomy followed by increased values without differences to the control group. These data highly suggest the presence of non-ovarian estradiol generation in ovariectomized animals. While the ovaries are the principal source of systemic estrogen in the pre-menopausal non-pregnant woman, estrogen biosynthesis beyond menopause is mainly peripheral, through conversion of androstendione or C19 steroids from the adrenal cortex. Other major sites of estrogen production like adipose tissue and skin, muscle and others have the capacity to convert C19 steroids to C18 steroids, but as they lack the ability to synthesize C19 precursors, they are dependent on circulating precursor C19 steroids for estrogen biosynthesis [38;39]. Sheep somehow seem to

compensate particularly well the estrogen deficiency, which probably is one of the reasons why ovariectomy only causes slight effects on ovine bone [30]. Further investigations are needed to determine the source of extragonadal extrogen. The ovariectomized sheep still might become a large animal model of postmenopausal osteoporosis, but reduction of non-ovarian estrogen production using aromatase inhibitors is indicated. Contrary to glucocorticoid excess, this inhibitor would simulate perfectly the mechanism of bone loss in postmenopausal women. In a study of Chavassieux, the administration of Lentaron, a specific inhibitor of the peripheral aromatase, during 6 months tended to amplify slightly the effects of ovariectomy on bone markers [21]. Because of seasonal variations and calcium intake, experiments in sheep should include all four seasons to minimize the seasonal fluctuations, and a calcium-wasting diet [25].

In summary, this study used a large variety of assessment methods to evaluate the long-term effect of ovariectomy in sheep in order to establish a large animal model of osteoporosis. Ovariectomy induced bone loss leading to decreased bone mineral density (-13%) and deterioration of microarchitecture. However, the destructive effect was limited to a few months and a rebound was observed in all treated animals. Our results suggest a short-term bone loss after ovariectomy in sheep probably due to extra-gonadal estrogen production. These data seems to indicate that ovariectomy in sheep does not simulate post-menopausal osteoporosis and are therefore not suitable for long term investigations.

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Figure 1

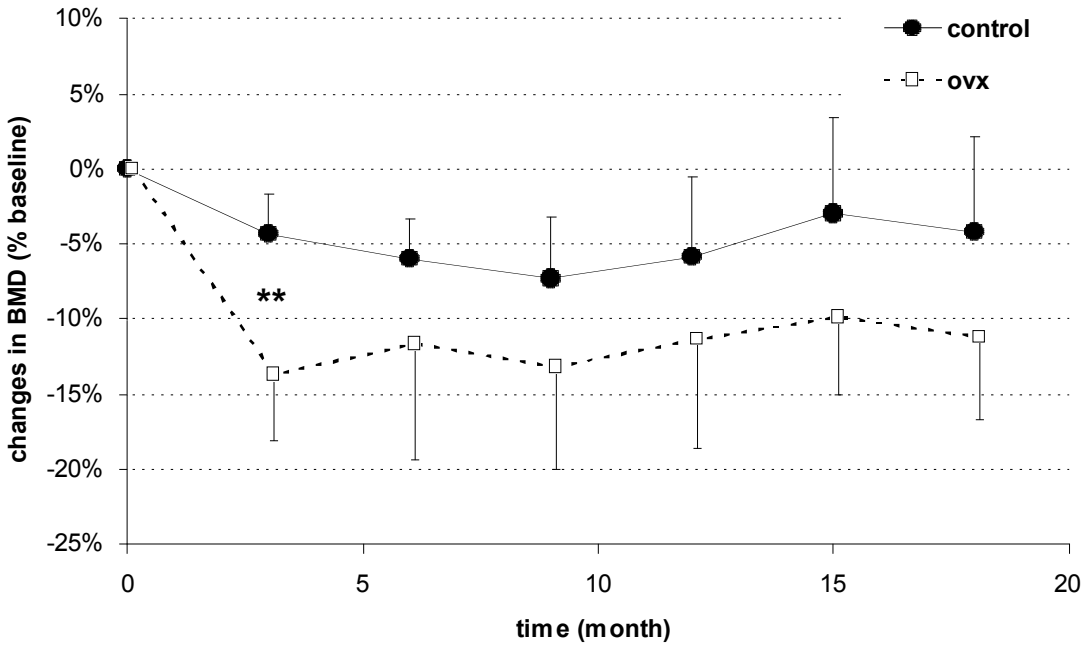

Figure 1: Cancellous bone mineral density at distal radius in control and ovariectomized sheep. Values are expressed as changes relative to baseline. **= $p<0.01$

Figure 2

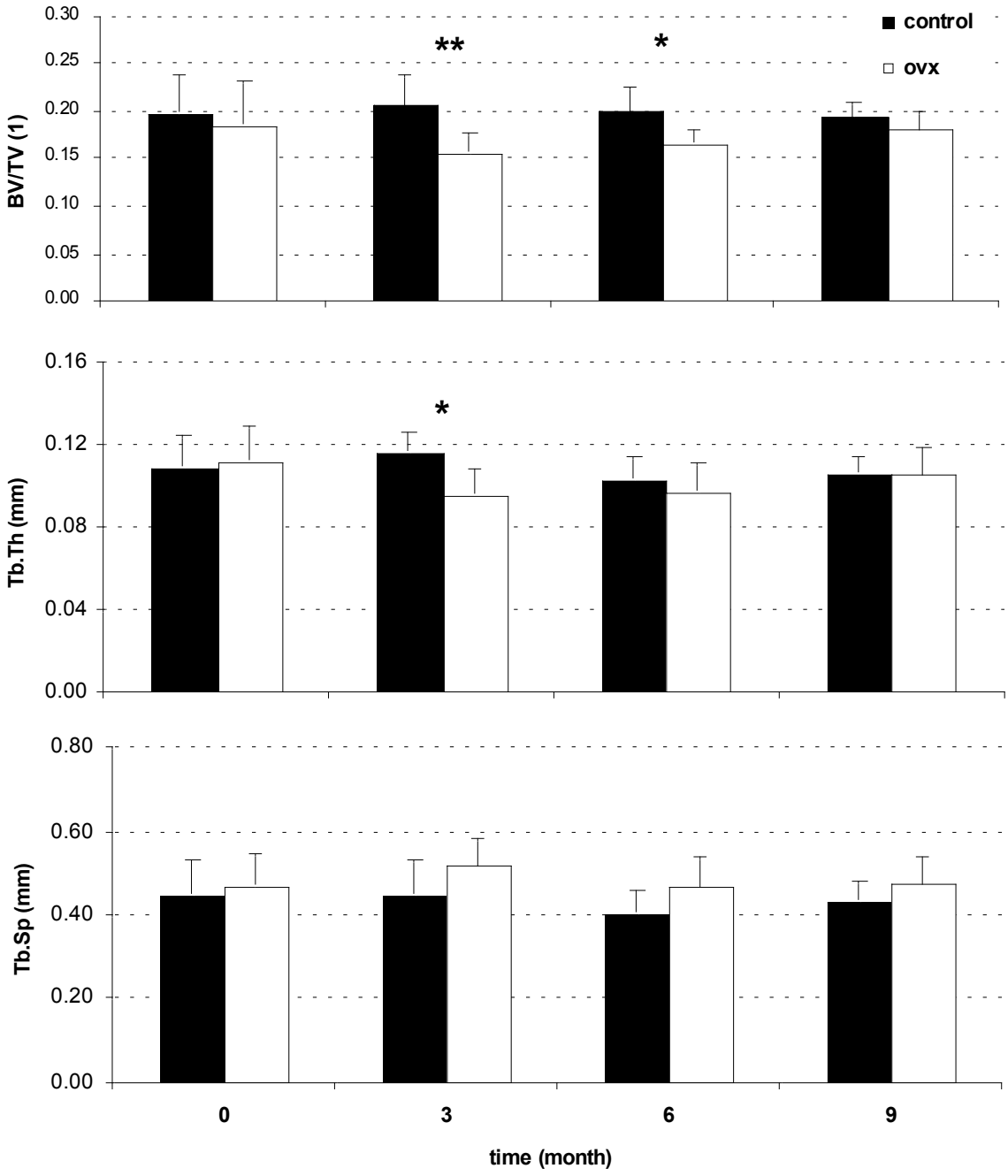

Figure 2: Structural parameters of cancellous bone at iliac crest biopsies in ovariectomized and control sheep. (BV/TV = bone volume / total volume; Tb.Th = trabecular thickness; Tb.Sp = trabecular separation). *= $p<0.05$; **= $p<0.01$.

Figure 3

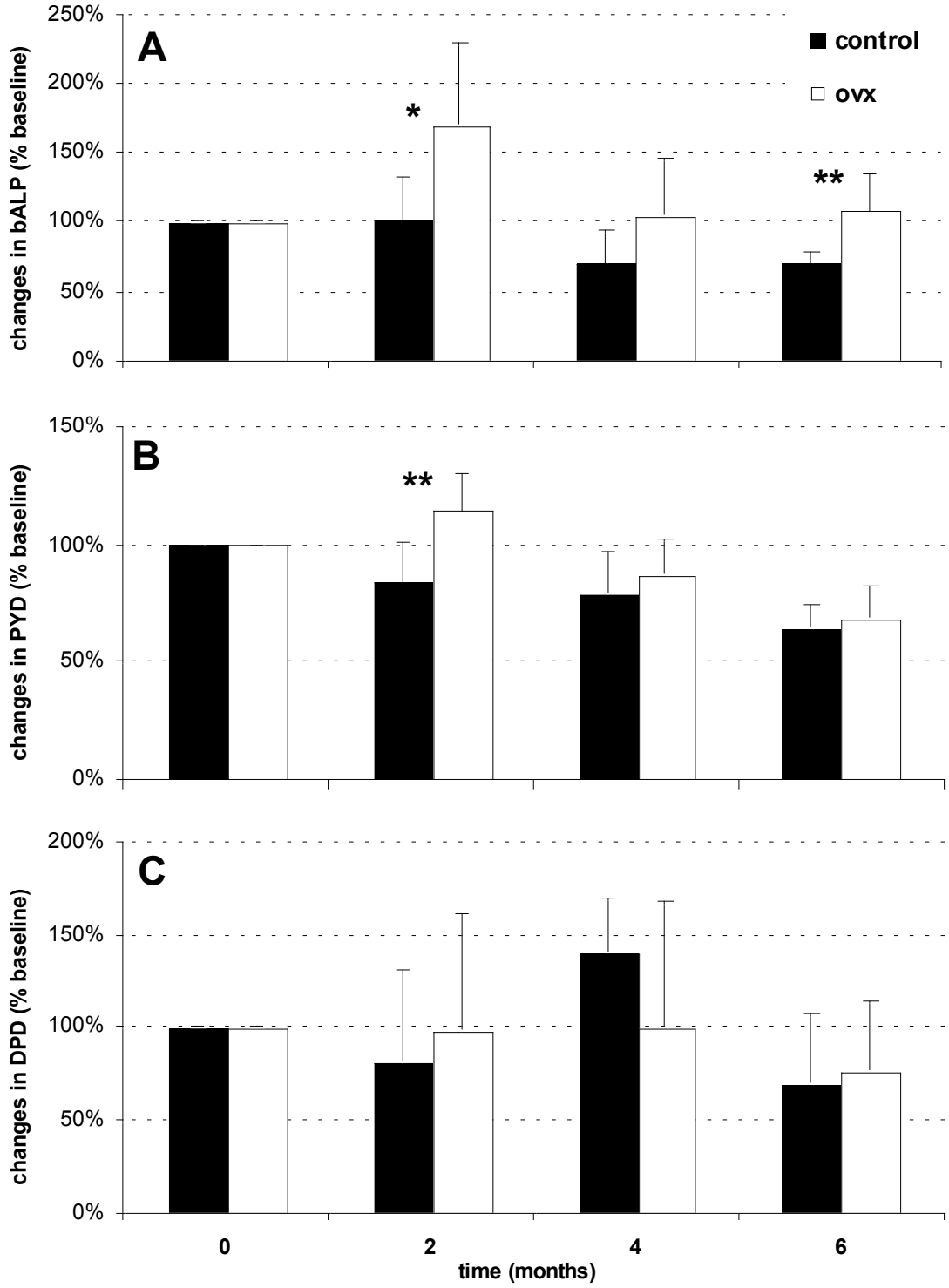

Figure 3: Marker of bone formation (A: serum bone alkaline phosphatase (bALP)) and resorption (B: serum pyridinoline (PYD) and C: urine deoxypyridinoline (DPD)). Values are expressed as changes relative to baseline (%). *= p<0.05; **= p<0.01.

Figure 4

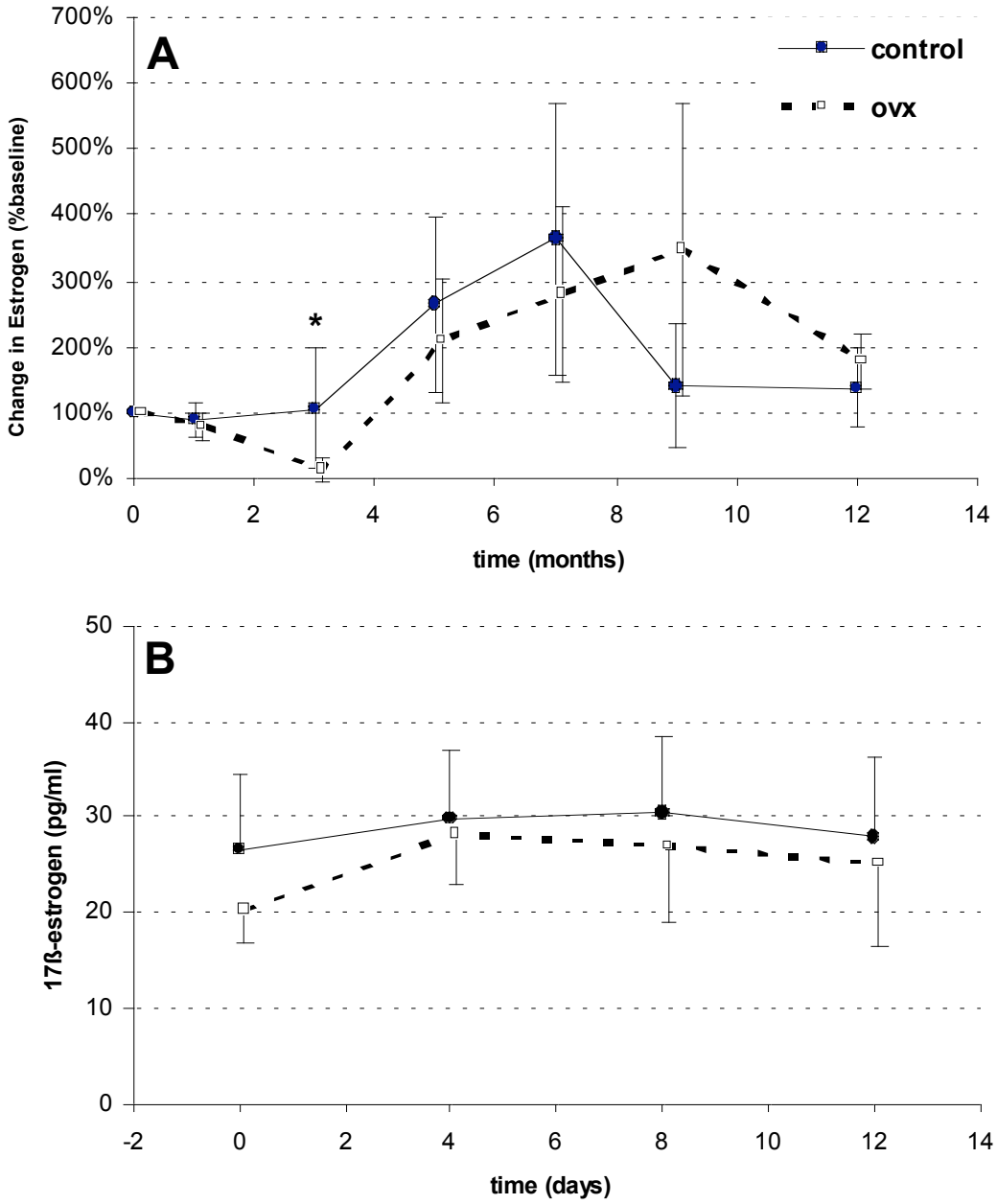

Figure 4: Serum levels of 17ß-estrogen over the course of 12 months (A) presented as changes relative to baseline values. At the end of the study estrogen was determined at short term intervals (B). *= $p<0.05$

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Curriculum vitae

Name	Ilonka, Sigrist
Geburtsdatum	29.12.1976
Geburtsort	Châtel-St-Denis
Nationalität	Schweizerin
Heimatort	Sarnen OW

1983- 1989 Primarschule Attalens

1989- 1992 Sekundarschule Châtel-St-Denis

1992- 1996 Gymnasium Bulle, Maturität Typ B

1996- 2001 Studium der Veterinärmedizin an der Universität Bern, Schweiz

2001 Eidgenössische Schlussprüfung für Tierärzte, Universität Bern, Schweiz

2002- 2004 Anstellung in gemischter Tierpraxis Jacober und Loepfe, Bazenheid, Schweiz

2004- 2005 Anstellung im AO-Forschungsinstitut, Davos, Schweiz

2005- 2007 Anstellung als Assistentin an der Grosstierklinik des Tierspitals der Universität Montréal, St-Hyacinthe, Qc, Kanada